Available online at www.sciencedirect.com

SCIENCE DIRECT

Developmental Biology 285 (2005) 496 – 507

DEVELOPMENTAL BIOLOGY

www.elsevier.com/locate/ydbio

Glycogen synthase kinase 3 controls endochondral bone development: Contribution of fibroblast growth factor 18

Ravi M. Kapadia, Anyonya R. Guntur, Martina I. Reinhold, Michael C. Naski*

Department of Pathology, University of Texas Health Science Center at San Antonio, 7703 Floyd Curl Dr., San Antonio, TX 78229-3900, USA

Received for publication 4 March 2005, revised 24 June 2005, accepted 25 July 2005
Available online 18 August 2005

Abstract

Glycogen synthase kinase 3 (GSK3) inhibits signaling pathways that are essential for bone development. To study the requirement for GSK activity during endochondral bone development, we inhibited GSK3 in cultured metatarsal bones with pharmacological antagonists. Interestingly, we find that inhibition of GSK3 strongly repressed chondrocyte and perichondrial osteoblast differentiation. Moreover, chondrocyte proliferation was inhibited, whereas perichondrial cell proliferation was stimulated. These results mirror the effects of fibroblast growth factor signaling (FGF), suggesting the FGF expression is induced. Indeed, we showed that (1) FGF18 expression is stimulated following inhibition of GSK3 and (2) GSK3 regulates FGF18 expression through the control of β-catenin levels. Stimulation of cultured metatarsal with FGF18 had similar effects on the differentiation and proliferation of chondrocytes and perichondrial cells as GSK3 repression. This suggests that the regulation of FGF18 expression is a major function of GSK3 during endochondral bone development. Consistent with this, we showed that the effect of GSK3 inhibition on chondrocyte proliferation is repressed in tissues lacking a receptor for FGF18, FGF receptor 3.

Keywords: Glycogen synthase kinase; FGF18; Bone; Cartilage; Chondrocyte; β-catenin

Introduction

Endochondral bone formation is an exquisitely controlled process requiring both chondrocyte and osteoblast differentiation. During this process, chondrocytes sequentially differentiate through a series of stages to eventually become hypertrophic chondrocytes. Cells in the surrounding perichondrium differentiate in step with the chondrocytes and eventually form osteoblasts, which secrete a collar of bone around the chondrocytes (Ballock and O'Keefe, 2003; Kronenberg, 2003; van der Eerden et al., 2003). The molecular events that control endochondral bone development are incompletely understood. However, genetic and biochemical studies have defined certain signaling and transcriptional pathways as essential for endochondral development. For example, Indian hedgehog is vital for chondrocyte and osteoblast differentiation (Lanske et al., 1996; St-Jacques et al., 1999; Vortkamp et al., 1996). Also, the transcription factor nuclear factor of activated T-cells (NFAT) performs important functions during cartilage gene expression and the differentiation of chondrocyte precursors (Tomita et al., 2002). Additionally, canonical Wnt signaling through β-catenin is required for normal chondrocyte and osteoblast differentiation (Akiyama et al., 2004; Guo et al., 2004; Hu et al., 2005). Significantly, each of these disparate pathways is regulated by a common kinase, glycogen synthase kinase 3 (GSK3).

GSK3 is a ubiquitously expressed kinase that regulates diverse cellular processes ranging from metabolism to cell fate specification (Cohen and Frame, 2001). Though first described as the kinase that regulates glycogen synthesis (Hemmings et al., 1981), GSK3 is now recognized to regulate developmental pathways including Wnt and hedgehog signaling pathways. Hedgehog signals through the

* Corresponding author. Fax: +1 210 567 4819.
E-mail address: naski@uthscsa.edu (M.C. Naski).

0012-1606/$ - see front matter © 2005 Elsevier Inc. All rights reserved.
doi:10.1016/j.ydbio.2005.07.029

smoothed/patched receptor complex and stimulates the activity of the transcription factor cubitus interruptus (Gli is the vertebrate ortholog). GSK3 inhibits hedgehog signaling by phosphorylating cubitus interruptus, thereby inducing conversion to a transcriptional repressor (Jia et al., 2002; Price and Kalderon, 2002). GSK3 also inhibits the transcription factor NFAT. Phosphorylation of the amino-terminal regulatory domain by GSK3 results in nuclear export of NFAT (Okamura et al., 2000), thereby inhibiting transcriptional activity. Wnt signaling is antagonized by GSK3 through phosphorylation of β-catenin. β-catenin phosphorylation results in ubiquitin-dependent degradation through the proteosome. Because each of these signaling cascades is essential for bone and cartilage differentiation, we hypothesize that GSK3 activity must be tightly regulated during endochondral bone development.

Unlike many kinases, GSK3 is constitutively active. Additionally, while many kinases are activated following stimulus-dependent phosphorylation, GSK3 is inactivated following phosphorylation. Several kinases including PKA, p70S6 kinase, AKT and others catalyze phosphorylation of a serine residue near the amino-terminus of GSK3 (Cross et al., 1995; Fang et al., 2000; Stambolic and Woodgett, 1994). This phosphoserine then binds to the active site and strongly inhibits GSK3 (Frame et al., 2001). Wnt signaling through disheveled also inhibits GSK3, although the mechanism of inhibition is not entirely clear. Therefore, a large number of signaling pathways and extracellular ligands all inhibit the activity of GSK3. Because GSK3 is a constitutive kinase that is exclusively regulated through repressor pathways, we reasoned that we could best understand the function of GSK3 in endochondral development by repressing GSK3 activity. To investigate the requirement for GSK activity during endochondral bone development, we used the pharmacological antagonists of GSK3, lithium and SB216763 (Coghlan et al., 2000; Stambolic et al., 1996). We find that GSK3 activity is essential for endochondral bone development. Inhibition of GSK3 with SB216763 repressed chondrocyte and osteoblast differentiation in cultured metatarsals from mouse embryo. Significantly, GSK3 repression induces fibroblast growth factor 18 (FGF18) expression and in turn, FGF18 repressed chondrocyte and osteoblast differentiation. These results suggest a pathway whereby GSK regulates endochondral bone development through the control of FGF18 expression. In support of this, we showed that effects on GSK3 on chondrocyte proliferation are absent in metatarsal lacking the FGF18 receptor, fibroblast growth factor receptor 3.

Materials and methods

Reagents

Reagents were purchased from suppliers as follows: SB216763 from Tocris (Cookson, MO); lithium chloride from Sigma (St. Louis, MO); FGF18 Peprotech (Rocky Hill, NJ).

Metatarsal cultures

Metatarsals were dissected from 15.5 day C57Bl/6 or FGF receptor 3 null embryos and cultured in serum free alpha MEM (Invitrogen) containing with 0.005 mg/ml ascorbic acid, 0.3 mg/ml L-glutamine, 0.05 mg/ml gentamicin, 1 mM β-glycerophosphate and 0.2% bovine serum albumin. The genotyping of FGF receptor 3 mice was done as previously described (Colvin et al., 1996). Tissue explants were grown at 37°C in a humidified 5% CO_2 incubator. To ensure that comparison was made between samples with equivalent states of differentiation, the tissues were maintained as right and left pairs from a given embryo and cultured in neighboring wells of a 24 well cluster plate. All comparisons were made using matched pairs. For histological analyses, the samples were paraffin embedded following fixation in 4% buffered paraformaldehyde for 1 h at room temperature.

Cell culture and transfection

MC3T3E1 (clone 4; Wang et al., 1999) was obtained from the ATCC (Manassas, VA), cultured in α-MEM containing 10% fetal bovine serum (Hyclone), 2 mM L-glutamine, penicillin G (100 units/ml) and streptomycin (100 μg/ml) at 37°C in a humidified 5% CO_2 incubator. All cells were transfected in triplicate using a modified calcium phosphate precipitate and assayed for luciferase or β-galactosidase activity as previously described (McEwen et al., 1999; Reinhold et al., 2004b). The FGF18 luciferase plasmid contained a 3.4 kb *Msc*I fragment of the mouse FGF18 gene (extending from approximately −3.3 kb relative to the transcriptional start site) cloned into pGL3basic (Promega).

Immunofluorescence

4 μm paraffin sections were warmed to 70°C for 10 min, then deparafinized and re-hydrated with a graded alcohol series. Antigen retrieval was done for 20 min in 10 mM pH 6.0 citrate buffer heated to 95°C prior to immersion of the slides. Thereafter, the slides were washed with phosphate buffered saline (PBS) and blocked in 1% fraction-V heat shock treated bovine serum albumin (Fisher), 5% normal goat or rabbit serum (Jackson ImmunoResearch Laboratories), 0.1% Triton X-100, in PBS. After 1 h, the blocking buffer was removed and replaced with 50 μl of blocking buffer containing the primary antibody (β-catenin Santa Cruz, sc7963 or N-cadherin, Transduction Labs, 610920) at a 1:200 dilution. Slides remained in the humidified chamber and incubated at 4°C overnight. The slides were washed three times with PBS containing 0.1% TX-100 and then blocked for 1 h at room temperature. Secondary

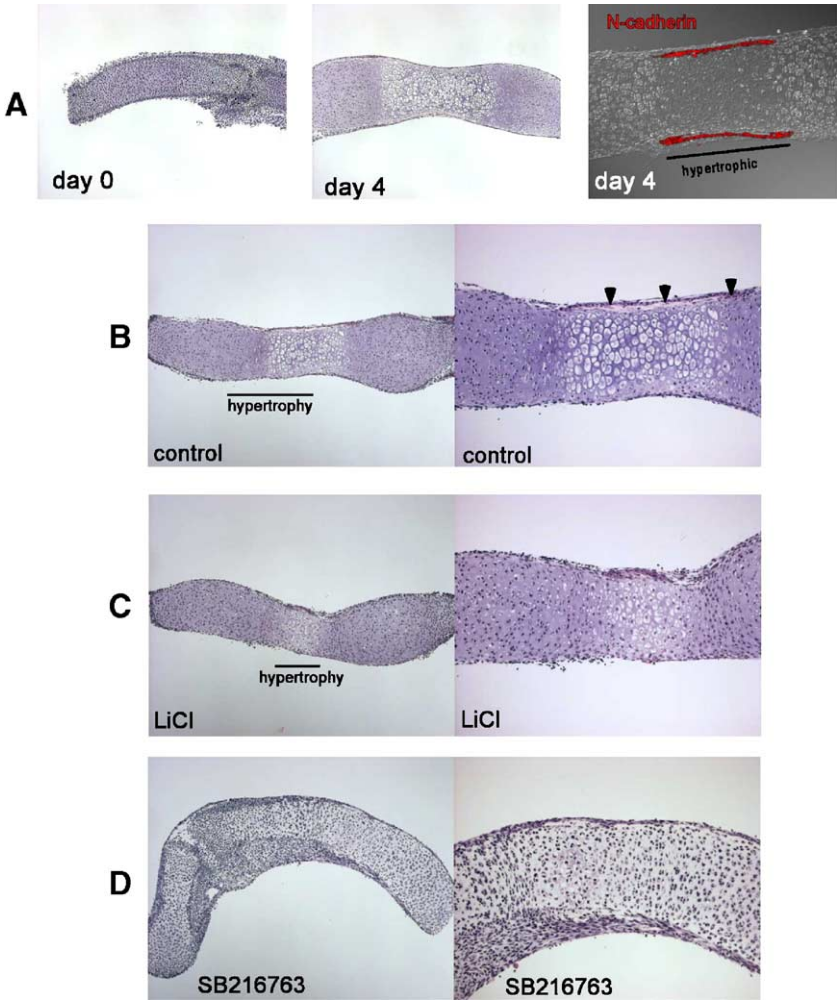
antibody was applied (CY3-goat anti-mouse, Jackson ImmunoResearch Laboratories) for 1 h at room temperature at a 1:800 dilution. The samples were washed three times in PBS and mounted with Pro-Long Anti-fade reagent (Molecular Probes). Images were obtained with a Zeiss Axioscope 2 fitted with 40× (NA 1.3) and 63× (NA 1.4) objectives. Deconvolution was done using Zeiss Axiovision software.

BrdU labeling

Tissues were pulse-labeled with 10 μM 5-bromo-2-deoxyuridine (Sigma) for 3 h. Tissues were then washed with PBS, paraffin embedded and sectioned to 4 μm. Slides were deparafinized, hydrated and immersed for 30 min in PBS containing 10% methanol and 3% hydrogen peroxide. After washing with PBS, the tissues were digested with 400 μg/ml pepsin (Sigma) containing 0.01 N HCl for 30 min at room temperature, neutralized for 10 min in 0.1 M borate pH 8.5, then rinsed with PBS. BrdU was detected with the Vectastain ABC kit (Vector Labs PK-4002) according to the manufacturer's protocol using an anti-BrdU antibody (Sigma B-2531). The labeling index was calculated by counting the number of BrdU-labeled nuclei and divided by the total number of cells within a grid drawn using Photoshop (Adobe). Equal areas of chondrocytes were counted in the control and treatment groups. For perichondrial cells, the entire perichondrium was counted. P values were determined using a paired Student's t test. The null hypothesis was rejected for $P < 0.05$.

Real time quantitative PCR

Total RNA was extracted by homogenizing metatarsals using RNA-Bee (Tel-Test, Inc.) according to the manufacturer's protocol. One microgram of RNA was reverse transcribed in 25 μl at 42°C for 2 h using TaqMan reverse transcription reagents with random hexamers according to manufacturer directions (Applied Biosystems). Quantitative PCR was performed in the ABI Prism 7000 Sequence Detection System according to manufacturer's directions (Applied Biosystems). 25 μl PCR reactions were made of SYBR Green 2× PCR Master Mix (Applied Biosystems), 5



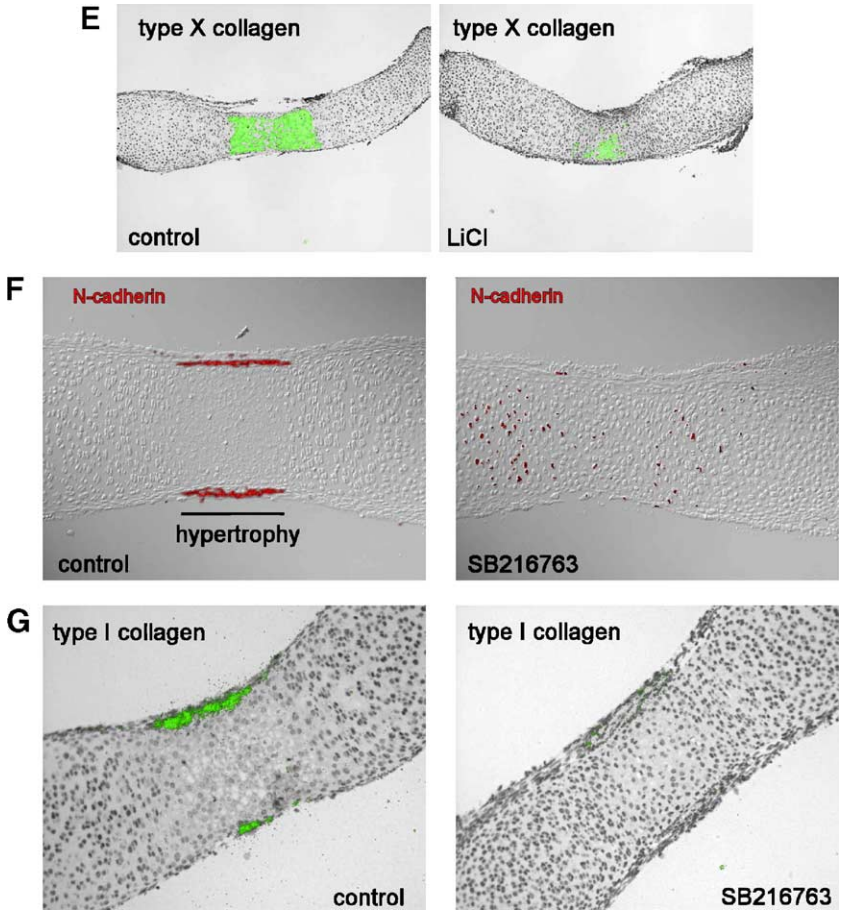


Fig. 1. Endochondral bone development in cultured mouse metatarsals. (A) 15.5 day mouse embryo metatarsals undergo development in culture. Left panel shows the tissue at the beginning of the culture period. Middle panel after 4 days in culture. Left panel shows that indirect immunofluorescence for N-cadherin identifies mature osteoblasts at sites of bone formation. Osteoblasts joined by N-cadherin containing adherens junctions develop in concert with chondrocyte hypertrophy. The figure shows the pseudocolored (red) fluorescence signal for N-cadherin overlaid on DIC images. (B) Control samples show the domain of chondrocyte hypertrophy and arrowheads indicate the osteoid bone collar. Right and left panels were obtained with 10× or 20× objectives, respectively. (C) Treatment of metatarsal cultures for 48 h with 15 mM lithium chloride repressed chondrocyte hypertrophy. Right and left panels were obtained with 10× or 20× objectives, respectively. (D) Chondrocyte hypertrophy is absent in metatarsal cultures treated for 48 h with 20 μM SB216763. Also note the absence of a bone collar. (E) In situ hybridization for collagen type X. Pseudo-colored (green) dark-field images are overlaid bright field images to show repressed chondrocyte hypertrophy in the sample treated with 15 mM lithium chloride. (F) Inhibition of GSK3 represses osteoblast differentiation. Indirect immunofluorescence for N-cadherin highlights osteoblasts at the site of bone collar synthesis adjacent to hypertrophic chondrocytes. N-cadherin linked osteoblasts are absent in the sample treated with 20 μM SB216763. The figure shows the pseudocolored (red) fluorescence signal overlaid on DIC images. (G) In situ hybridization for collagen type I. Pseudo-colored (green) dark-field images are overlaid bright field images to show repressed osteoblast differentiation in the sample treated with 20 μM SB216763. The figures show representative results from 4 independent experiments.

pmol of each forward and reverse primer and 3 μl of cDNA. Cycling conditions were 50°C for 2 min, 95°C for 10 min followed by 40 cycles of 15 s at 95°C and 60 s at 60°C. Primers were designed using PrimerExpress (Applied Biosystems). Oligonucleotides used were as follows: FGF1- 5′-CGGCTCGCAGACACCAA-3′, 5′-ACCAGTTCTTCTCCGCATGCT-3′; FGF2- 5′-GGACCCCAAGCGGCTCTA-3′, 5′-CCTCTCTCTTCTGCTTGGAGTTGT-3′; FGF3- 5′-GTGAACGGCAGCCTTGAGA-3′, 5′-CAGGTACCGCCCAGAAAAGA-3′; FGF4- 5′-CGAGGCGTGGTGAGCATCT-3′, 5′-CGTTGTAGTTGTTGGGCAGAAGT-3′; FGF7- 5′-TGAAGAACAGCTACAACATCATGGA-3′, 5′-TCAGTTCTTTGAAGTTGCAATCCT-3′; FGF8- 5′-CTCATTGTGGAGACCGATACTTTTG-3′, 5′-GCCGTTGCTCTTGGCAATT-3′; FGF9- 5′-ATCTTCCCCAACGGTACTATCCA-3′; 5′-CTCGTTCATGCCGAGGTAGAGT-3′; FGF10- 5′-AGTGCGGGAAGGCATGTG-3′, 5′-CTCCGATTTCCACTGATGTTATCTC-3′; FGF11- 5′-TCCTTCACCCACTTCAATCTGA-3′, 5′-TGAAATGTGGCGAGCTGTACA-3′; FGF12- 5′-AACCCCAGCTGAAAGGGATT-3′, 5′-GCCACCACACGCAGTCCTA-3′; FGF13- 5′-AGGAGACCAGAGCCTCAGCTT-3′, 5′-CGTCTTTGGTGCCATCAATG-3′; FGF14- 5′-GCTCTCGATGGAACCAAGGA-3′, 5′-TTGCTATGTACAACCCTGTCTTCAC-3′; FGF18- 5′-ACTGCTGTGCTTCCAGGTTC-3′, 5′-CCCAGGACTTGAATGTGCTT-3′; FGF20- 5′-GGATCACAGTCTCTTCGG-

TATCCT-3′, 5′-TTTGTCATTCATCCCAAGGTACAG-3′; FGF22- 5′-CTTCTCCTCCACTCACTTTTTCCT-3′; 5′-GCCTGAGTACACAGCTTTGATCAC-3′; aggrecan- 5′-ACTGCAGCGATGACCCTC-3′, 5′-GGAATCCCTAGCTGCTTCG-3′; collagen II- 5′-GAAGGTGGAAAGCAAGGTGA-3′, 5′-CATCAGTACCAGGAGTGCCA-3′; conduction- 5′-AAAACGGATTCAGGTCCTTCAA-3′; 5′-GTCAGTGCGTCGCTGGATAAC-3′; Indian hedgehog- 5′-CAATCCCGACATCATCTTCA-3′; 5′-GCGGCCCTCATAGTGTAAAG-3′.

In situ hybridization

4 μm sections of paraffin embedded tissues were processed, hybridized and washed according to the mRNA locator protocol (Ambion). The FGF18 (gift from D. Ornitz, St. Louis, MO) and collagen type X (B. Olson Boston, MA) riboprobes were labeled as previously described (Naski et al., 1998).

Results

To understand how GSK3 controls endochondral bone formation, we studied the differentiation of mouse embryo metatarsals in organ culture. This is an established method for investigating the differentiation of chondrocytes and osteoblasts during endochondral bone development (Dieudonne et al., 1994; Serra et al., 1999). Mouse metatarsal bone rudiments were prepared from 15.5 day embryos and cultured in serum-free medium. At the time of isolation, the bone rudiments contain immature chondrocytes surrounded by a sheath of undifferentiated perichondrial cells (Fig. 1A). Osteoblasts and hypertrophic chondrocytes are absent. After 3–4 days in serum-free medium, chondrocyte hypertrophy begins (Fig. 1A). Simultaneously, cells in the perichondrium differentiate into osteoblasts and secrete a collar of woven bone around the hypertrophic chondrocytes. The mature osteoblasts synthesizing the collar of bone are identified by the presence of N-cadherin organized into adherens junctions (Fig. 1A).

To investigate how GSK3 regulates endochondral bone formation in metatarsals, we used specific pharmacological antagonists of GSK3. GSK3 is a constitutive kinase that is regulated through repressor pathways. Therefore, we modeled the regulation of GSK3 using the pharmacological inhibitors lithium and SB216763. Interestingly, endochondral bone development was strikingly inhibited following inhibition of GSK3 with either lithium or SB216763 for 48 h. This was evidenced by substantial or complete inhibition of chondrocyte hypertrophy in the samples treated with the GSK3 inhibitors lithium and SB216763 (Figs. 1B–D). And is further evidenced by the repression of type X collagen expression, a marker for hypertrophic chondrocytes (Fig. 1E). In addition to the inhibition of chondrocyte differentiation, we observed a concomitant inhibition of perichondrial osteoblast differentiation. This was evidenced by the absence of a collar of woven bone surrounding the hypertrophic chondrocytes (compare the right panels of Figs. 1B and D), the absence of osteoblasts containing N-cadherin organized into adherens junctions (Fig. 1F) and dramatically reduced expression of type I collagen (Fig. 1G). Note that scattered chondrocytes contain detectable levels of N-cadherin following treatment with SB216763. Immature chondroblasts express N-cadherin (Oberlender and Tuan, 1994a,b). The presence of N-cadherin in these cells reflects the strong repression of differentiation and immature phenotype of these cells.

To further characterize the effects of GSK3 on chondrocyte differentiation, we performed quantitative RT-PCR with RNA extracted from metatarsals. Corroborating the in situ hybridization data of Fig. 1E, inhibition of GSK3 repressed the expression of type X collagen greater than 3000-fold as measured by quantitative real time PCR (data not shown). Also, other chondrocyte-specific genes were inhibited. The expression of both type II collagen and aggrecan was dramatically repressed following inhibition of GSK3 (Fig. 2). Also, the expression of Indian hedgehog and patched, the hedgehog receptor, was repressed in these samples. Because patched is both a receptor and transcriptional target of Indian hedgehog, these findings suggest that hedgehog signaling was repressed. Notice that the expression of conductin, a transcriptional target of β-catenin (Jho et al., 2002), was induced following inhibition of GSK3.

To investigate if GSK3 also regulated cell proliferation, we performed bromodeoxyuridine (BrdU) labeling experiments. Metatarsal samples were treated with the

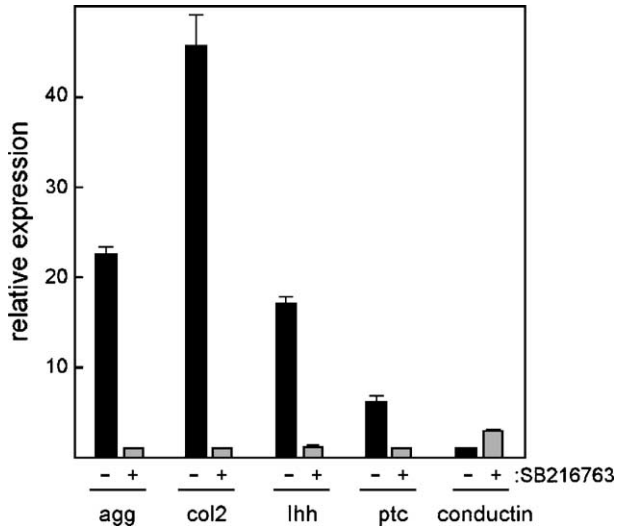


Fig. 2. Real time RT-PCR measurement of gene expression in cultured metatarsals. Relative expression determined by quantitative real time PCR of the aggrecan (agg), type II collagen (col2), Indian hedgehog (Ihh), patched (ptc) and conductin. RNA harvested from metatarsals cultured 48 h in the presence or absence of 20 μM SB216763 was reverse transcribed with random hexamers. Expression levels were normalized to the 18s rRNA internal control. The expression levels for each pair are relative to each other with the lesser sample defined as one. The results show representative results of two independent samples.

GSK3 inhibitor SB216763 and thereafter pulsed with BrdU for 3 h. Proliferating cells were identified by immunostaining for BrdU. Interestingly, inhibition of GSK3 had opposite effects on chondrocyte and perichondrial cell proliferation (Fig. 3). Inhibition of GSK3 repressed chondrocyte proliferation (Fig. 3B), whereas perichondrial cell proliferation was stimulated following inhibition of GSK3 (Fig. 3C). Significantly, these data showing inhibition of chondrocyte differentiation and proliferation resemble the effects of FGF signaling in cartilage (Deng et al., 1996; Kato and Iwamoto, 1990; Li et al., 1999; Naski et al., 1998) and are similar to results obtained when bone explants are stimulated with FGF (Mancilla et al., 1998).

Because these results suggest augmented FGF signaling, we hypothesized that inhibition of GSK3 stimulates FGF expression. To test this, we determined FGF expression by quantitative real time PCR. Interestingly, we observed that FGF13 and FGF18 were substantially induced following the inhibition of GSK3 (Fig. 4A). The induction of FGF18 is intriguing because FGF18 is an essential regulator of endochondral bone development (Liu et al., 2002; Ohbayashi et al., 2002). To further define the effects of GSK3 on FGF expression, we performed in situ hybridization for FGF18. Consistent with the quantitative PCR results, FGF18 expression was induced following treatment with the GSK3 inhibitor, SB216763 (Figs. 4B and C). Significantly, FGF18 expression is most abundant in the perichondrial cells. This coincides with perichondrial regions wherein β-catenin levels are strongly upregulated adjacent to the hypertrophic chondrocytes in samples without the GSK3 inhibitor (Fig. 4D). Because inhibition of GSK3 by SB216763 results in stabilization of β-catenin (βcat) and increased levels of βcat protein (data not shown), we hypothesized that βcat is essential for induction of FGF18 expression. To determine if βcat regulates the transcription of FGF18, we performed experiments using the immature osteoblast cell line MC3T3E1. Fig. 5A shows that inhibition of GSK3 induces FGF18 in MC3T3E1 cells. To determine if βcat-dependent transcription is activated in response to inhibition of GSK3, we used TOPFLASH, a luciferase reporter requiring β-catenin/TCF for activation (Korinek et al., 1997). We found that TOPFLASH activity is stimulated in a dose-dependent manner by SB216763 (Fig. 5B). To determine if the FGF18 promoter is stimulated by βcat, cells were transfected with a luciferase reporter containing 3.4 kb of the proximal FGF18 promoter. Significantly, the FGF18 promoter was induced when βcat-dependent activity was stimulated by either inhibition of GSK3 with SB216763 or co-transfection of a stabilized form of βcat (Figs. 5C and D). These data support the conclusion that inhibition of GSK3 induces FGF18 gene expression through upregulation of βcat levels.

We showed that inhibition of GSK3 stimulated FGF expression and repressed chondrocyte differentiation and proliferation. To determine if these findings may be linked, we investigated whether the effects of FGF18 on metatarsal rudiments phenocopy the effects of GSK3 inhibition. Interestingly, we observed similarities between inhibition of GSK3 and activation of FGF signaling with FGF18.

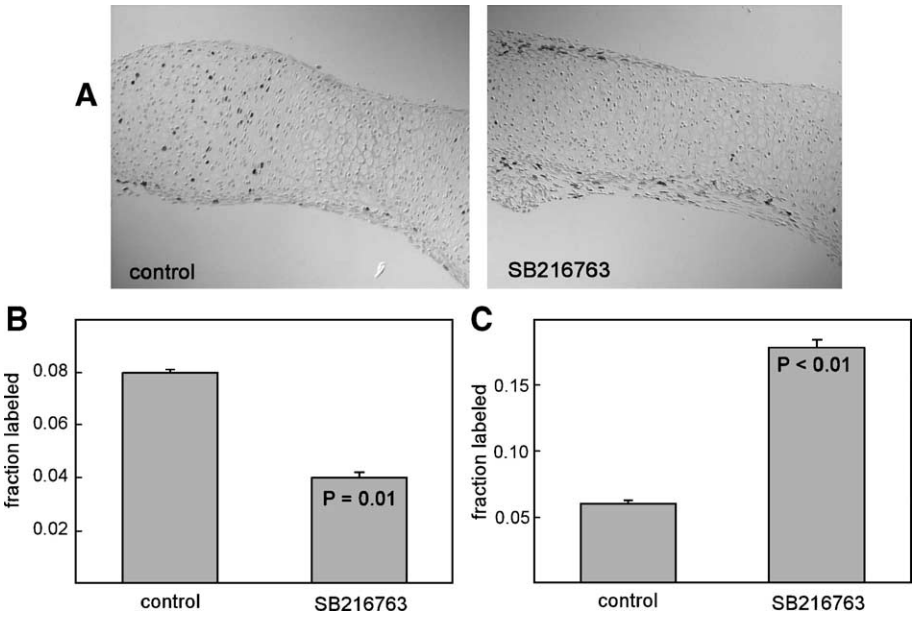


Fig. 3. Inhibition of GSK3 inhibits chondrocyte proliferation, but augments perichondrial cell proliferation. (A) DIC images of BrdU immunohistochemistry showing labeling of chondrocyte and perichondrial cells from control (left panel) or 20 μM SB216763 (right panel) treated samples following 48 h treatment. (B) Chondrocyte proliferation after treatment with 20 μM SB216763 for 48 h as measured by the fraction of BrdU-labeled chondrocyte nuclei. The null hypothesis was rejected with $P = 0.01$. (C) Perichondrial cell proliferation after treatment with 20 μM SB216763 for 48 h as measured by the fraction of BrdU-labeled nuclei. The null hypothesis was rejected with $P < 0.01$. The figures show representative results from 3 independent experiments.

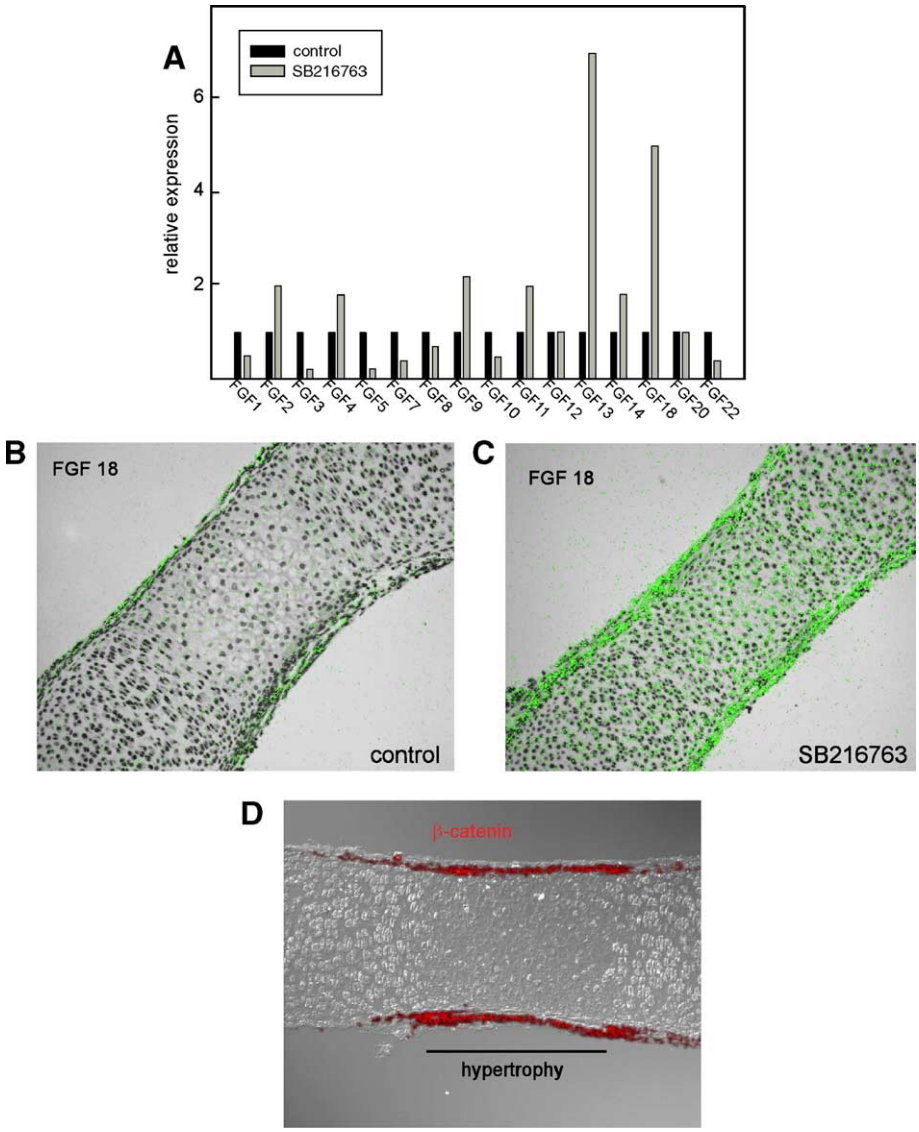


Fig. 4. Inhibition of GSK3 stimulates FGF18 expression. (A) Survey of FGF expression by real time quantitative RT-PCR. Relative FGF expression using RNA isolated from samples cultured 48 h in the presence or absence of 20 μM SB216763. FGF expression levels were normalized to the 18s rRNA internal control. Expression levels for each pair (with or without SB216763) are relative to the control sample (defined as one). The results show representative results of two independent samples. (B and C) In situ hybridization for FGF18 in metatarsals cultured in the presence or absence of 20 μM SB216763 for 48 h. Results show the overlay of pseudocolored (green) dark-field images on bright field images. (D) Indirect immunofluorescence of β-catenin. The fluorescence signal for β-catenin is overlaid to the DIC image showing upregulation of beta-catenin in the perichondrial layer. Sample was cultured without SB216763.

Stimulation of metatarsals with FGF18 inhibits both chondrocyte proliferation and differentiation. This is demonstrated by the inhibition of chondrocyte hypertrophy and inhibition of BrdU labeling (Figs. 6A and B). Also, perichondrial osteoblast differentiation is concomitantly inhibited as evidenced by diminished synthesis of a collar of woven bone (Fig. 6A), the absence of mature osteoblasts joined by N-cadherin-containing adherens junctions (Fig. 6C) and strong repression of type I collagen expression (Fig. 6D). Also, FGF18 stimulated perichondrial cell proliferation (Fig. 6B, right), similar to inhibition of GSK3.

These findings suggest that the repression of endochondral bone development resulting from inhibition of GSK3 is in part caused by the induction of FGF expression. To better understand the role of FGF signaling following repression of GSK3, we performed experiments with metatarsals from mice lacking FGF receptor 3 (FGFR3−/−). FGF receptor 3 is expressed in cartilage and is the major receptor for FGF18 in cartilage. Therefore, if the induction of FGF18 contributes to the inhibition of endochondral development, then the inhibition will be diminished in the cartilage of FGFR3 null mice. Consistent with this, we find that in the absence of FGFR3 chondrocyte proliferation was not repressed when GSK3 is inhibited. Fig. 7A shows that as observed previously chondrocyte proliferation is inhibited when GSK3 is inhibited in heterozygous mice. However,

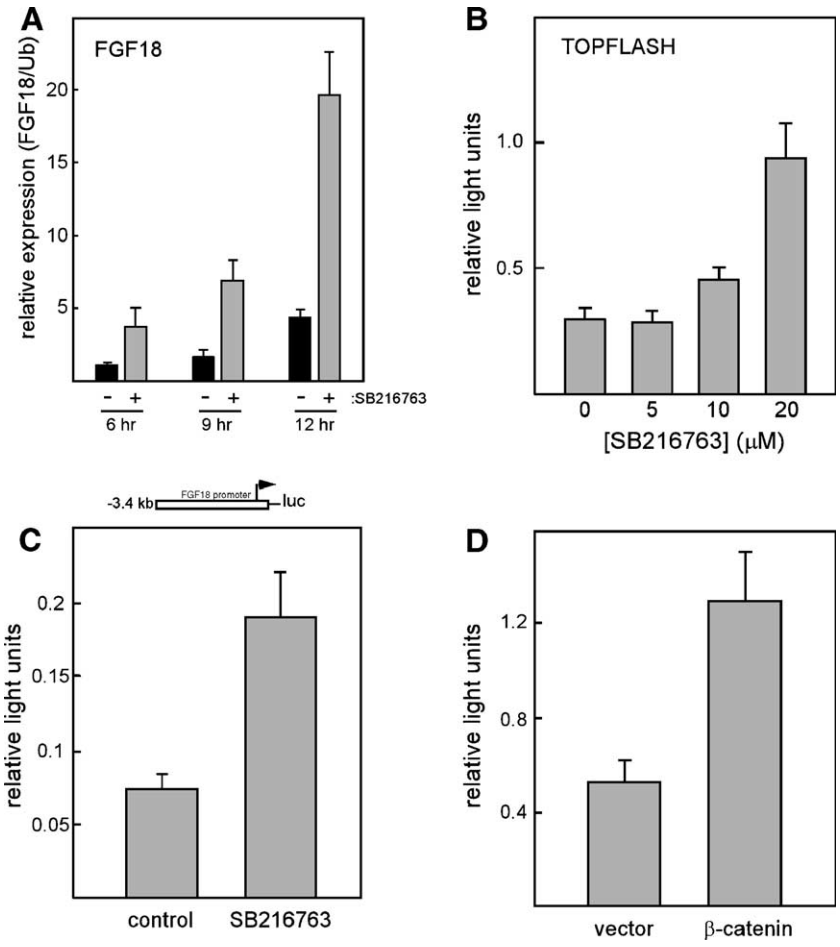


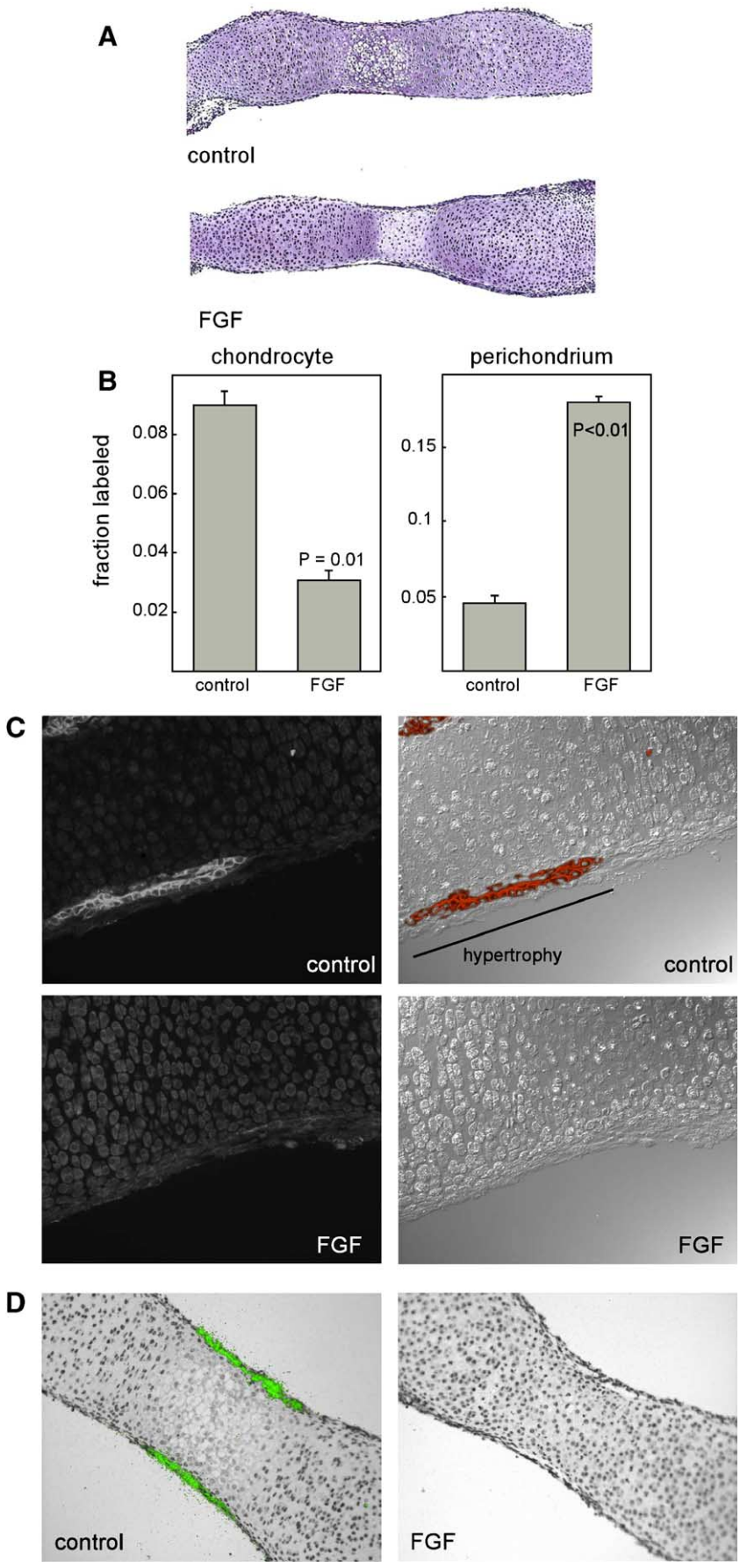
Fig. 5. Induction of FGF18 expression. (A) Induction of FGF18 expression in MC3T3E1 immature osteoblasts after inhibition of GSK3 with 18 μM SB216763 for the indicated times. Expression levels were determined by real time RT-PCR. (B) Activation of TOPFLASH luciferase in MC3T3E1 osteoblasts after treatment with the indicated concentrations of SB216763 for 24 h. (C) Inhibition of GSK3 stimulates the FGF18 luciferase promoter in MC3T3E1 osteoblasts. MC3T3E1 osteoblasts were transiently transfected with the FGF18 luciferase promoter as indicated above the panel and treated for 24 h with 20 μM SB216763. Luciferase activity was normalized to β-galactosidase activity derived from a constitutive co-transfected plasmid. (D) β-catenin activates the FGF18 promoter. MC3T3E1 osteoblasts were transiently transfected with the FGF18 luciferase promoter and a proteosome resistant form of βcat. Luciferase activity was normalized to β-galactosidase activity derived from a constitutive co-transfected plasmid. Results are representative of three independent experiments.

inhibition of GSK3 does not repress chondrocyte proliferation in metatarsals from FGFR3−/− mice.

Discussion

Our data have shown that GSK3 activity is essential for endochondral bone development. Inhibition of GSK3 by either lithium or SB216763 strongly inhibits chondrocyte and perichondrial osteoblast differentiation. Because these agents inhibit GSK3 kinase activity by different mechanisms yet similarly repress endochondral bone development, we conclude that the effects of these inhibitors are due to specific inactivation of GSK3 (Coghlan et al., 2000; Ryves and Harwood, 2001). The specificity of SB216763 has been validated by evaluation of a large panel of kinases (Coghlan et al., 2000).

Because GSK3 activity is essential for endochondral bone development, certain substrates of GSK3 are vital regulators of endochondral bone development. There are many putative substrates for GSK3 (Woodgett, 2001); however, several well-recognized substrates are central regulators of bone development. The hedgehog signaling pathway activates the transcription factor Gli. Recent data show that the *Drosophila* ortholog of Gli, cubitus interruptus, is phosphorylated by GSK3 and that this leads to the repression of Gli (Jia et al., 2002; Price and Kalderon, 2002). Because hedgehog (Indian hedgehog) is expressed in cartilage, we would predict extra Gli activity when GSK3 is inhibited. However, our data suggest less rather than more Gli activity. Patched is a transcription target of Gli (Goodrich et al., 1996) and therefore if Gli activity is stimulated patched expression should increase when GSK3 is inhibited. However, we showed that patched expression is reduced, suggesting reduced rather than increased Gli activity. Our data showing repression of Ihh expression following treatment with the GSK3 inhibitor are consistent with less Gli activity. Therefore, we conclude


A
control
FGF
B
chondrocyte
perichondrium
fraction labeled
0.08
0.06
0.04
0.02
P = 0.01
0.15
0.1
0.05
P<0.01
control
FGF
control
FGF
C
control
hypertrophy
control
FGF
FGF
D
control
FGF

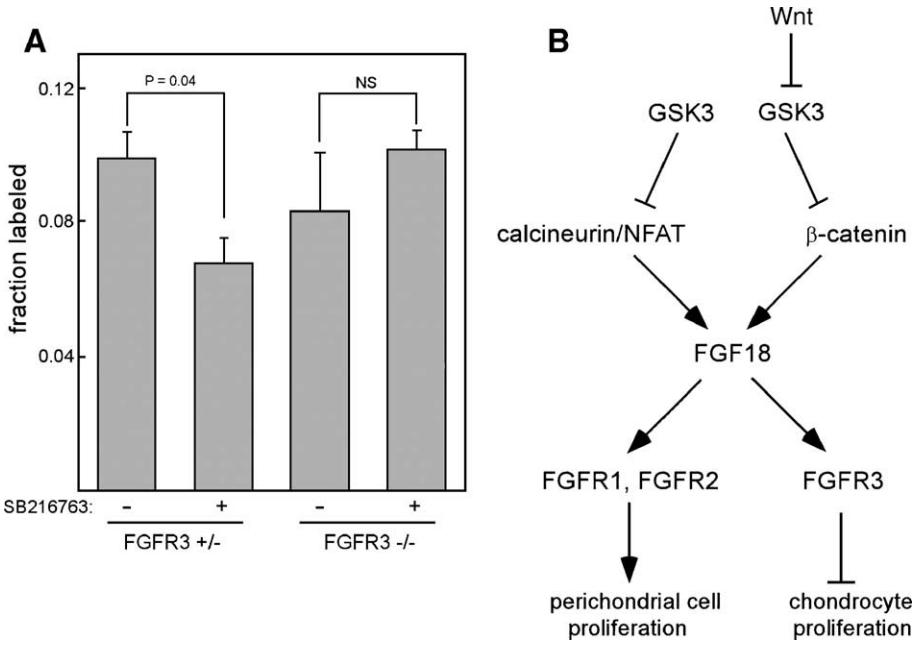


Fig. 7. (A) Inhibition of GSK3 does not repress proliferation of FGFR3−/− chondrocytes. Metatarsals were dissected from FGFR3 heterozygous (+/−) or null (−/−) embryos and cultured in the presence of absence of 20 μM SB216763 for 48 h. Thereafter, the samples were pulsed with BrdU and processed for immunohistochemistry. The null hypothesis was rejected for samples from heterozygous, but not null mice. Results are representative of two independent experiments. (B) Working model for the regulation of FGF18 expression. Inhibition of GSK3 through canonical Wnt signaling stimulates FGF18 expression via upregulation of β-catenin. Inhibition of GSK3 also promotes FGF18 expression by maintaining calcineurin-dependent NFAT activity. The induction of FGF18 expression represses chondrocyte proliferation through the action of the principle fibroblast growth factor receptor of cartilage, FGFR3. Simultaneously, perichondrial cell proliferation is stimulated following activation of fibroblast growth factor receptors 1 and 2 (FGFR1).

that Gli does not appear to be the dominant substrate of GSK3 in these experiments. The inhibition Ihh expression may contribute to the regulation of osteoblast differentiation. Osteoblast differentiation is absent in Ihh−/− mice (Chung et al., 2001; St-Jacques et al., 1999), suggesting that the reduction of Ihh expression following inhibition of GSK3 contributes to the repression of osteoblast differentiation.

The transcription factors, nuclear factor of activated T-cells are also GSK3 substrates that may regulate chondrocyte differentiation, given that we previously showed that NFAT stimulates chondrogenesis (Tomita et al., 2002). Because phosphorylation of NFAT by GSK3 inhibits NFAT activity (Beals et al., 1997; Neal and Clipstone, 2001), we hypothesize that NFAT activity is augmented in metatarsals wherein GSK3 is inhibited. In published work, we show that FGF18 expression is induced through NFAT-dependent pathways (Reinhold et al., 2004a). Significantly, we now show that FGF18 is induced in response to GSK3 inhibition. This suggests that NFAT may be an important substrate of GSK3 during endochondral bone development. However, NFAT transcriptional activity requires an additional calcium-dependent stimulus that dephosphorylates NFATs via the protein phosphatase calcineurin. We showed that TGFβ induces FGF18 activation through a calcium-dependent pathway (Reinhold et al., 2004a). In addition, data showing that TGFβ represses chondrocyte differentiation during endochondral ossification suggest that a TGFβ family member may be induced in metatarsal bone rudiments (Bohme et al., 1995; Dieudonne et al., 1994; Serra et al., 1999). Therefore, we investigated if TGFβ expression is induced by GSK3 inhibition. We did not observe changes in the expression of TGFβ1, 2 or 3 by QPCR (data not shown). However, our analyses of RNA extracted from the entire metatarsal may not detect a significant induction of TGFβ in a discreet population of cells. Further studies will be required to determine if TGFβ or another pathway such as the Wnt-calcium pathway can stimulate NFAT activity in developing endochondral bone. Interestingly, NFAT activ-

Fig. 6. Effects of FGF18 on endochondral development. (A) H & E stained sections of metatarsals treated for 48 h with 800 ng/ml FGF18 (bottom panel). (B) Effects of FGF18 on chondrocyte (left panel) and perichondrial cell (right panel) proliferation. Samples were treated with 800 ng/ml FGF18 for 48 h, pulsed with BrdU and then processed for immunohistochemistry. The fraction of labeled nuclei was measured and the null hypothesis was rejected at the indicated *P* values. (C) Repression of osteoblast differentiation after treatment with 800 ng/ml FGF18. Mature osteoblasts are detected by immunofluorescence for N-cadherin. The control samples (top) show N-cadherin immunofluorescence in cells adjacent to hypertrophic chondrocytes. Left panels show the fluorescence signal. Right panels show the pseudocolored signal overlaid to the DIC image. FGF18 treated samples (bottom) lack N-cadherin signals via immunofluorescence. (D) In situ hybridization for collagen type I. Pseudo-colored (green) dark-field images are overlaid bright field images to show repressed osteoblast differentiation in the sample treated with 800 ng/ml FGF18.

ity can be stimulated by Wnt signaling (Saneyoshi et al., 2002), therefore, Wnt-calcium signaling is a potential NFAT activator.

βcat is a GSK3 substrate that also has dramatic effects on bone and cartilage differentiation. Phosphorylation of βcat by GSK3 causes degradation by the proteosome. Wnt signaling represses proteolysis of βcat by inhibiting GSK3. Therefore, the inhibition of GSK3 by SB216763 can simulate the effects of Wnt signaling during endochondral development. The effects of Wnt signaling on chondrocytes are complex. Both inhibition and stimulation of differentiation have been observed. Forced expression of Wnt-5a in developing chicken limbs slowed the maturation of chondrocytes as evidenced by diminished expression of type X collagen (Hartmann and Tabin, 2000). Similar results are obtained when either Wnt5a or Wnt5b is expressed in cartilage of mice (Yang et al., 2003). Surprisingly, Wnt5a null mice show the same phenotype of delayed chondrocyte differentiation as mice overexpressing Wnt5a (Yang et al., 2003). Moreover, Wnt 4 expression accelerates chondrocyte hypertrophy (Hartmann and Tabin, 2000). These data show that Wnts can both inhibit and stimulate endochondral bone development. Forced expression of Wnt14 in chondrocytes of mice caused small, dysplastic skeletons, joint fusions and cartilage hypertrophy was strongly repressed (Hartmann and Tabin, 2001). Similar observations were seen when a stabilized form of βcat was overexpressed in cartilage implying that the inhibition of endochondral bone development is via canonical Wnt signaling (Akiyama et al., 2004; Guo et al., 2004). Surprisingly, however, cartilage-specific deletion of βcat in chondrocytes also causes formation of a dysplastic skeleton with joint fusions and slowed chondrocyte differentiation (Akiyama et al., 2004; Guo et al., 2004; Hu et al., 2005). While these data indicate that a single simple conclusion cannot account for all effects of Wnt signaling on endochondral bone development, our results support a model wherein the upregulation of βcat levels represses chondrocyte differentiation. Also, while this manuscript was under review, other reports provided evidence for inhibitory effects of Wnt signaling on chondrocyte differentiation (Day et al., 2005; Hill et al., 2005). Significantly, we showed that the expression of FGF18 is induced following inhibition of GSK3. Moreover, we showed that FGF18 is induced through a βcat-dependent pathway. Others have similarly found that FGF18 is induced by βcat (Shimokawa et al., 2003). Based on these data and our published data showing that FGF18 is induced through calcium-dependent pathways requiring calcineurin/NFAT activation, we propose that FGF18 is induced via a bipartite pathway when GSK3 is repressed. One pathway simulates canonical Wnt signaling whereby FGF18 is induced after upregulation of βcat protein levels. The second path stimulates FGF18 through calcineurin/NFAT activation. This model and the effects of FGF18 on cell proliferation in bone rudiments are summarized in Fig. 7B.

FGF18−/− mice show features of advanced differentiation including an enlarged hypertrophic region and increased chondrocyte proliferation (Liu et al., 2002; Ohbayashi et al., 2002). These results indicate that FGF18 inhibits endochondral differentiation. This is further evidenced by our results showing that FGF18 repressed chondrocyte and osteoblast differentiation and inhibited chondrocyte proliferation. These data mirror the effects on GSK3 inhibition on cultured metatarsals. Given our data showing that FGF18 is induced following GSK3 inhibition, we conclude that GSK3 is an essential regulator of FGF18 expression and that FGF18 is an important downstream mediator of the effects of GSK3 in endochondral bone development. We showed that FGF18 expression is induced by βcat, suggesting that canonical Wnt signaling induced FGF18. Interestingly, the induction of FGF expression through Wnt signaling is essential at several steps of skeletal development. For example, the anatomical site of limb initiation is determined by a Wnt-FGF signaling relay. Wnt2b is expressed in the intermediate mesenchyme of the developing embryo and induces FGF10 expression in the adjacent lateral plate mesenchyme. This relay determines the site for limb development as demonstrated by ectopic limbs that result from forced expression of Wnt2b (Kawakami et al., 2001). Later during limb development, FGF8 is induced in response to Wnt3 in the ectoderm of nascent limb buds (Barrow et al., 2003; Kawakami et al., 2001). This step is critical for both the formation and stability of the apical ectodermal ridge. We propose that the expression of FGF18 in response to βcat is another example of a conserved and essential relay whereby Wnt signaling induces FGF expression during skeletal development.

Acknowledgments

This work was supported by NIH grants AR47070, AR050024 and the Paul Beeson Physician Faculty Scholars in Aging Research Program.

References

Akiyama, H., et al., 2004. Interactions between Sox9 and beta-catenin control chondrocyte differentiation. Genes Dev. 18, 1072–1087.

Ballock, R.T., O'Keefe, R.J., 2003. The biology of the growth plate. J. Bone Jt. Surg. Am. 85-A, 715–726.

Barrow, J.R., et al., 2003. Ectodermal Wnt3/beta-catenin signaling is required for the establishment and maintenance of the apical ectodermal ridge. Genes Dev. 17, 394–409.

Beals, C.R., et al., 1997. Nuclear export of NF-ATc enhanced by glycogen synthase kinase-3. Science 275, 1930–1934.

Bohme, K., et al., 1995. Terminal differentiation of chondrocytes in culture is a spontaneous process and is arrested by transforming growth factor-beta 2 and basic fibroblast growth factor in synergy. Exp. Cell Res. 216, 191–198.

Chung, U.I., et al., 2001. Indian hedgehog couples chondrogenesis to osteogenesis in endochondral bone development. J. Clin. Invest. 107, 295–304.

Coghlan, M.P., et al., 2000. Selective small molecule inhibitors of glycogen synthase kinase-3 modulate glycogen metabolism and gene transcription. Chem. Biol. 7, 793–803.
Cohen, P., Frame, S., 2001. The renaissance of GSK3. Nat. Rev., Mol. Cell Biol. 2, 769–776.
Colvin, J.S., et al., 1996. Skeletal overgrowth and deafness in mice lacking fibroblast growth factor receptor 3. Nat. Genet. 12, 390–397.
Cross, D.A., et al., 1995. Inhibition of glycogen synthase kinase-3 by insulin mediated by protein kinase B. Nature 378, 785–789.
Day, T.F., et al., 2005. Wnt/beta-catenin signaling in mesenchymal progenitors controls osteoblast and chondrocyte differentiation during vertebrate skeletogenesis. Dev. Cell 8, 739–750.
Deng, C., et al., 1996. Fibroblast growth factor receptor 3 is a negative regulator of bone growth. Cell 84, 911–921.
Dieudonne, S.C., et al., 1994. Opposite effects of osteogenic protein and transforming growth factor beta on chondrogenesis in cultured long bone rudiments. J. Bone Miner. Res. 9, 771–780.
Fang, X., et al., 2000. Phosphorylation and inactivation of glycogen synthase kinase 3 by protein kinase A. Proc. Natl. Acad. Sci. U. S. A. 97, 11960–11965.
Frame, S., et al., 2001. A common phosphate binding site explains the unique substrate specificity of GSK3 and its inactivation by phosphorylation. Molecular Cell 7, 1321–1327.
Goodrich, L.V., 1996. Conservation of the hedgehog/patched signaling pathway from flies to mice: induction of a mouse patched gene by Hedgehog. Genes Dev. 10, 301–312.
Guo, X., et al., 2004. Wnt/beta-catenin signaling is sufficient and necessary for synovial joint formation. Genes Dev. 18, 2404–2417.
Hartmann, C., Tabin, C.J., 2000. Dual roles of Wnt signaling during chondrogenesis in the chicken limb. Development 127, 3141–3159.
Hartmann, C., Tabin, C.J., 2001. Wnt-14 plays a pivotal role in inducing synovial joint formation in the developing appendicular skeleton. Cell 104, 341–351.
Hemmings, B.A., et al., 1981. Purification of glycogen synthase kinase 3 from rabbit skeletal muscle. Copurification with the activating factor (FA) of the (Mg-ATP) dependent protein phosphatase. Eur. J. Biochem. 119, 443–451.
Hill, T.P., et al., 2005. Canonical Wnt/beta-catenin signaling prevents osteoblasts from differentiating into chondrocytes. Dev. Cell 8, 727–738.
Hu, H., et al., 2005. Sequential roles of Hedgehog and Wnt signaling in osteoblast development. Development 132, 49–60.
Jho, E.H., et al., 2002. Wnt/beta-catenin/Tcf signaling induces the transcription of Axin2, a negative regulator of the signaling pathway. Mol. Cell. Biol. 22, 1172–1183.
Jia, J., et al., 2002. Shaggy/GSK3 antagonizes Hedgehog signalling by regulating Cubitus interruptus. Nature 416, 548–552.
Kato, Y., Iwamoto, M., 1990. Fibroblast growth factor is an inhibitor of chondrocyte terminal differentiation. J. Biol. Chem. 265, 5903–5909.
Kawakami, Y., et al., 2001. WNT signals control FGF-dependent limb initiation and AER induction in the chick embryo. Cell 104, 891–900.
Korinek, V., et al., 1997. Constitutive transcriptional activation by a beta-catenin-Tcf complex in APC−/− colon carcinoma. Science 275, 1784–1787.
Kronenberg, H.M., 2003. Developmental regulation of the growth plate. Nature 423, 332–336.
Lanske, B., et al., 1996. PTH/PTHrP receptor in early development and Indian hedgehog-regulated bone growth. Science 273, 663–666.
Li, C.L., et al., 1999. A Lys644Glu substitution in fibroblast growth factor receptor 3 (FGFR3) causes dwarfism in mice by activation of STATs and ink4 cell cycle inhibitors. Hum. Mol. Genet. 8, 35–44.
Liu, Z., et al., 2002. Coordination of chondrogenesis and osteogenesis by fibroblast growth factor 18. Genes Dev. 16, 859–869.
Mancilla, E.E., et al., 1998. Effects of fibroblast growth factor-2 on longitudinal bone growth. Endocrinology 139, 2900–2904.
McEwen, D.G., et al., 1999. Fibroblast growth factor receptor 3 gene transcription is suppressed by cyclic adenosine 3′,5′-monophosphate: identification of a chondrocytic regulatory element. J. Biol. Chem. 274, 30934–30942.
Naski, M.C., et al., 1998. Repression of hedgehog signaling and BMP4 expression by fibroblast growth factor receptor 3 in growth plate cartilage of transgenic mice. Development 125, 4977–4988.
Neal, J.W., Clipstone, N.A., 2001. Glycogen synthase kinase-3 inhibits the DNA binding activity of NFATc. J. Biol. Chem. 276, 3666–3673.
Oberlender, S.A., Tuan, R.S., 1994a. Expression and functional involvement of N-cadherin in embryonic limb chondrogenesis. Development 120, 177–187.
Oberlender, S.A., Tuan, R.S., 1994b. Spatiotemporal profile of N-cadherin expression in the developing limb mesenchyme. Cell Adhes. Commun. 2, 521–537.
Ohbayashi, N., et al., 2002. FGF18 is required for normal cell proliferation and differentiation during osteogenesis and chondrogenesis. Genes Dev. 16, 870–879.
Okamura, H., 2000. Concerted dephosphorylation of the transcription factor NFAT1 induces a conformational switch that regulates transcriptional activity. Mol. Cell 6, 539–550.
Price, M.A., Kalderon, D., 2002. Proteolysis of the Hedgehog signaling effector Cubitus interruptus requires phosphorylation by glycogen synthase kinase 3 and casein kinase 1. Cell 108, 823–835.
Reinhold, M.I., et al., 2004a. FGF18 represses noggin expression and is induced by calcineurin. J. Biol. Chem. 279, 38209–380219.
Reinhold, M.I., et al., 2004b. Fibroblast growth factor receptor 3 gene: regulation by serum response factor. Mol. Endocrinol. 18, 241–251.
Ryves, W.J., Harwood, A.J., 2001. Lithium inhibits glycogen synthase kinase-3 by competition for magnesium. Biochem. Biophys. Res. Commun. 280, 720–725.
Saneyoshi, T., et al., 2002. The Wnt/calcium pathway activates NF-AT and promotes ventral cell fate in *Xenopus* embryos. Nature 417, 295–299.
Serra, R., et al., 1999. Parathyroid hormone-related peptide (PTHrP)-dependent and -independent effects of transforming growth factor beta (TGF-beta) on endochondral bone formation. J. Cell Biol. 145, 783–794.
Shimokawa, T., et al., 2003. Involvement of the FGF18 gene in colorectal carcinogenesis, as a novel downstream target of the beta-catenin/T-cell factor complex. Cancer Res. 63, 6116–6120.
Stambolic, V., Woodgett, J.R., 1994. Mitogen inactivation of glycogen synthase kinase-3 beta in intact cells via serine 9 phosphorylation. Biochem. J. 303, 701–704.
Stambolic, V., et al., 1996. Lithium inhibits glycogen synthase kinase-3 activity and mimics wingless signalling in intact cells. Curr. Biol. 6, 1664–1668.
St-Jacques, B., et al., 1999. Indian hedgehog signaling regulates proliferation and differentiation of chondrocytes and is essential for bone formation. Genes Dev. 13, 2072–2086.
Tomita, M., et al., 2002. Calcineurin and NFAT4 induce chondrogenesis. J. Biol. Chem. 277, 42214–42218.
van der Eerden, B.C., et al., 2003. Systemic and local regulation of the growth plate. Endocr. Rev. 24, 782–801.
Vortkamp, A., et al., 1996. Regulation of rate of cartilage differentiation by Indian hedgehog and PTH-related protein. Science 273, 613–622.
Wang, D., et al., 1999. Isolation and characterization of MC3T3-E1 preosteoblast subclones with distinct in vitro and in vivo differentiation/mineralization potential. J. Bone Miner. Res. 14, 893–903.
Woodgett, J.R., 2001. Judging a protein by more than its name: GSK-3. Sci. Signal Transduct. Knowl. Environ. 2001, RE12.
Yang, Y., et al., 2003. Wnt5a and Wnt5b exhibit distinct activities in coordinating chondrocyte proliferation and differentiation. Development 130, 1003–1015.